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Novel Metalloprotease Production by Solid State Fermentation of Agroindustrial Residues: A Statistical Approach for Process Optimization

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ABSTRACT:
Enzyme production by bioprocess provides the best use of the underutilized agro-industrial waste. Biological pathways involving agricultural waste for the synthesis of enzymes contribute to the perpetuation of environment and making the overall process economically manageable. Two step optimization was employed for the optimization of protease production by a newly isolated strain *Bacillus cereus* B80 using rice bran as substrate in solid state fermentation. Plackett- Burman and centre composite design in response surface methodology were used to build statistical models to screen out the significant variables, and then study the effect of identified three significant variables on enzyme production. The fermentation variables screened with the Plackett Burman design were substrate concentration, particle size, inoculum size, moisture ratio, incubation time and chemical components as K_2HPO_4, KH_2PO_4, KNO_3, NaCl, $MgSO_4$ and $CaCl_2$. Three significant variables substrate concentration, time and NaCl were selected on the basis of their $E(x_i)$ values and pareto chart and studied for their interactive effect on the enzyme production via central composite design. The maximum protease production after optimization was 4818 U/g after 84h when the substrate concentration was 25% and NaCl concentration was 0.65% exhibiting an overall 2.5 fold increase as compared with the production of 1955 U/g of enzyme with basal medium.

Keywords: Metalloprotease, *Bacillus* sp., Solid state fermentation, Plackett–Burman design, Response surface methodology, Centre composite design

INTRODUCTION

Worldwide demand for industrial enzymes is expected to achieve levels of US$3.74 billion by 2015 [1]. Proteases constitute the largest product segment of about 60-65% with applicability in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery [2]. The latest advancements in this field are production of chemicals, enzymes and food/feed materials using biological entities like microorganisms [3]. Moreover, these biological products are naturally sustainable, produced in mild operating conditions, with fewer greenhouse gas emissions than other processing aids by utilization of cheap renewable resources. They cut the manufacturing cost by reducing energy and water consumption; lessen impact on environment by decreasing the need for harsh chemical additives and lowering the amount of waste by-products [4]. The cost of the substrate affects the overall economy when it comes to bulk production of commodities like enzymes. Approximately 40% of the production cost of industrially important enzymes is estimated to derive from the cost of growth medium [5].

Solid state fermentation has evolved in the recent years as a technology that utilizes the solid waste substrates as agricultural residues for the production of commercial enzymes as they contain high moisture and provide readily available energy and nitrogen source [6,7]. This technology offers numerous advantages over submerged fermentation (SmF), including high titers and large volumes of production, simple fermentation equipments, less effluent generation, easy process management, low cost substrates and low energy requirements over submerged fermentation techniques [8,9]. Rice is the second largest crop produced around the world contributing 27.3 million tons of rice bran production across the globe [10]. The utilization of rice bran as substrate in SSF processes provides an alternative avenue and value-addition to these residues.

Different parameters as type of substrate, substrate concentration, solid moisture ratio, contact time and the interrelation amongst them affect the overall process and success of SSF. Optimization hence, becomes a central dogma in a biotechnological process. Among various nonlinear and quadratic optimization techniques available, response surface methodology is the most studied and employed

technique used for optimization process in the recent years [11,12,13]. Response-surface methodology is a multivariate analysis tool comprising mathematical and statistical techniques for generating empirical models that includes factorial design and regression analysis and evaluates the effects of the individual factors and provides optimal levels of variables for desirable responses [14]. Second-order models as central composite designs (CCDs), box benkhem where the least variations could turn into a major factor in over all commercial success of the production process design, Factorial CCD used in RSM have many advantages as they can take on a wide variety of functional forms and this flexibility allows them to more closely approximate the true response surface [15].

The present study demonstrates the optimization of production parameters of a novel metalloprotease by utilization of agro-industrial residues in solid state fermentation. The enzyme exhibits thermal and pH stability in a wide range of temperature (30-60^{o}C) and pH (6-9) with maximum activity at 60°C and pH 8.0. The increase in enzyme activity in presence of metal ions and nearly complete inhibition by EDTA, DTT and β-mercaptoethanol indicates that it is a metalloproteases [16]. Keeping in view the importance the wide applicability of the enzyme, the factors contributing to the enzyme production were statistically optimized by employing Plackett Burman and central composite design under RSM.

MATERIALS AND METHODS

Microorganism

Protease producing *Bacillus* strain was selected from microbial culture collection available in the laboratory characterized and identified by Saxena and Singh [16].

Preparation of Inoculum

The selected strain was inoculated in nutrient broth (containing (g/l) peptone-5; beef extract -3; NaCl-5) and incubated at 37^{o}C for 16h at 120rpm. A standard inoculum with 3.2 x 10^{8} cfu/ml (0.5 OD at 600nm) was obtained.

Substrate

Five types of agro industrial waste as gram husk, wheat bran, rice bran, mustard oilseed cake and soybean cake were procured from the local mills and processed to obtain a uniform size of about 2-4 mm.

Solid State Fermentation

The initial SSF experiments were performed in the basal media containing (%) substrate-5; K_2HPO_4 -0.3; KH_2PO_4- 0.1; KNO_3-0.1; NaCl 0.8; $MgSO_4$-0.05; $CaCl_2$- 0.01 with particle size 2-4 mm, inoculum size 1%, moisture ratio 1:4 for 72h. The experiments were carried out in 500ml Erlenmeyer flasks containing substrate moistened with sterile liquid nutrient medium containing chemical components. The contents of the flasks were mixed thoroughly, autoclaved at 121^{o}C for 15min at 15psi. After cooling the flasks were inoculated with the prepared inoculum, and incubated at 37^{o}C for the desired period. The flask without any inoculation with the microorganism was taken as a control, and assayed for any enzyme activity.

Enzyme Extraction

For enzyme extraction phosphate buffer (0.1 M), pH 7 was added to the fermentation product, stirred and mixed thoroughly. The mixture was passed through whatman filter paper (No-1, Millipore) and the culture filtrates obtained were centrifuged at 10000 rpm for 15 min. The clear supernatant obtained was used as the crude enzyme.

Enzyme Assay

Protease activity was measured using casein as substrate [17]. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg tyrosine per ml in 1 min under the experimental conditions used. The experiments were carried out in triplicates and standard error was calculated.

Optimization of Enzyme Production Parameters

Selection of Substrate

Substrate for the SSF experiment was selected by using one factor at a time technique. Gram husk, wheat bran, rice bran mustard oilseed cake and soybean cake were each used substrate in the basal media. The substrate that gave the best enzyme production was chosen for further optimization along with other production factors.

Statistical Optimization of Production Parameters

The statistical optimization of enzyme production parameters was performed in two steps. In the first step screening of the variables that significantly affected the production was performed by Plackett Burman design, while the second step optimization of the screened variables was performed by central composite design. Design Expert® 8.0.2.0 (Stat-Ease,

Inc., Minneapolis, MN, USA) was used to design and analyze both the experiments.

Plackett Burman design for screening of Significant factors

Plackett Burman is a two-level fractional factorial design that screens out the main factors affecting a process with the least number of experiments [12,18]. Eleven parameters including physical factors as substrate concentration, particle size, inoculum size, moisture ratio, incubation time and chemical components as K_2HPO_4, KH_2PO_4, KNO_3, NaCl, $MgSO_4$ and $CaCl_2$ were involved in the production of protease enzyme. The 12 run PB design was used to study these factors where each variable was examined in two levels, −1 for low level and +1 for high level [15,19] as shown in Table 1. The effect of each variable was determined by the following equation:

$$E(xi) = 2(\Sigma Mi + – Mi –) / N$$

Where E(xi) is the concentration effect of the tested variable, Mi+ and Mi– are the total production from the trials where the measured variable (xi) was present at high (+) and (-) low concentrations, respectively; and N is the number of trials.

Central composite design for second level optimization

RSM combines statistical experimental designs and empirical model building by regression for the purpose of process optimization. Based on the results obtained from the PB design, three factors were selected for the second level optimization employing CCD under response surface methodology A total of 20 trials were employed with each factor varying over 6 levels – 5 alpha points (-1.682 +1.682), and an axial point located at a specified distance a from the design center in each direction. In this design the alpha points (+/-) allow the evaluation of all main factors and their interaction effects, while the purpose of the center/axial points is to estimate the pure error and curvature.

The relationships among the variables can be expressed mathematically in the form of a second order quadratic polynomial model equation

$$Y=\beta o+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3$$

where Y is the measured response, β_0 is the intercept term, β_1, β_2, β_3 are linear coefficient, β_{11}, β_{22}, β_{33} are quadratic coefficient, β_{12}, β_{13}, β_{23} are interaction coefficient and X_1, X_2 X_3 are coded independent variables.

RESULTS

Microorganism

The newly isolated strain *Bacillus cereus* B80 (GenBank accession number JQ040533) used in the study produces a novel Mn^{+2} activated metalloprotease.

Selection of the solid substrate

Five different types of agricultural wastes were checked for enzyme production by SSF. The maximum enzyme production was observed with rice bran (1955.63 U/g), followed by mustard oilseed cake (1084 U/g). Gram Husk, soybean cake and wheat bran exhibited low enzyme production (138, 438 and 506 U/g of enzyme respectively) in similar conditions (Fig 1).

Statistical Optimization of media

The significant variables effecting the enzyme production were screened out by Plackett Burman design and in the second step optimization of significant variables on enzyme production along with their interaction was performed.

Screening of significant factors by Plackett Burman design

Plackett Burman design is a widely used two factorial optimization step employed for screening of significant variables affecting a response. The design matrix involving the selected variables and their corresponding responses are shown in Table 1a. $E(x_i)$ value of the variables investigated is represented in Table 1b. A large $E(x_i)$ coefficient, either positive or negative, indicates a large impact on response; while a coefficient close to zero indicates little or no effect. The $E(x_i)$ values show that substrate concentration, time, moisture ratio, inoculum size, NaCl, $MgSO_4$ and $CaCl_2$ had positive effect on the enzyme production, while particle size, KH_2PO_4, K_2HPO_4 and KNO_3 showed negative $E(x_i)$ values.

The pareto graph is a important tool for choosing the statistically significant factors after the analysis of the effects of all the parameters on the response. It identifies those factors that have the greatest cumulative effect on the system, and thus screen out the less significant factors. It is represented by a series of bars whose heights reflect the frequency or impact of factors. In the present analysis, the "t-values" of time, NaCl, substrate concentration,

KNO_3, moisture content, inoculums size and moisture content, were significantly above the Bonferroni Limit (14.7818), showing that these were the most significant factors affecting the enzyme production (Fig 2).

The Model F-value of 150.00 as calculated via ANOVA (Table 2) implies the model is significant with factors A, B, D, E, F, H, L (Prob > F" less than 0.0500) as significant model terms. The regression value of the model is 0.9985. The "Pred R-Squared" of 0.9467 is in reasonable agreement with the "Adj R-Squared" of 0.9919. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of our model is 42.008 which indicates an adequate signal. This model can be used to navigate the design space.

Thus based on the $E(x_i)$ values, pareto chart, and ANOVA, three most significant factors substrate concentration, time and NaCl were selected for second level optimization by CCD. Other positively affecting factors were taken at their maximum limit, negatively effecting factors KH_2PO_4, K_2HPO_4 and KNO_3 were eliminated and particle size was taken in their lower limit.

Central composite design for second level optimization

In the second step of optimization, substrate concentration, time and NaCl were optimized by RSM. The design summary, matrix and the corresponding responses of CCD experiments along with the actual, predicted values and the residual are shown in (Table 3a & b).

The data obtained for the response protease production was subjected to analysis of variance (ANOVA). The result of the ANOVA is represented in Table 4.

The Model F-value of 340.34 implies the model is significant. A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms for protease production. The "Lack of Fit F-value" of 3.05 implies the Lack of Fit is not significant. Non-significant lack of fit is good , this shows that the model is fit.

The regression equation coefficients were calculated and the data was fitted to a second-order polynomial equation. Thus the response (Y), (in terms of coded factors) protease production by the selected *Bacillus* sp. can be expressed in terms of the following regression equation:

Protease Activity = +4753.24+291.10A + +131.49B + 103.14C -181.69AB – 803.44AC +228.94BC – $814.47A^2 – 959.52B^2 – 316.23C^2$

where A is substrate, B is moisture and C is inoculum size.

The regression equation obtained from ANOVA (Table 4) showed that the multiple correlation coefficient (R^2) was 0.9967 (a value >0.75 indicates fitness of the model). The "Pred R-Squared" of 0.9801 is in reasonable agreement with the "Adj R-Squared" of 0.9938. Adeq Precision ratio 49.405 of our model indicates an adequate signal. This model can be used to navigate the design space.

Three-dimensional response surface contour graphs were constructed by plotting the response (protease production) on the Z-axis against any two independent variables, while maintaining third variable at its optimal level.

Fig 3(a) shows an increase in protease production with increase in NaCl and time at a constant substrate concentration, but further increase in these two factors resulted in concomitant decline of the response.

Similarly the response enhanced by increasing the time and substrate concentration when NaCl concentration was fixed (Fig 3c). Fig 3a and 3c exhibit a fairly strong degree of curvature of 3D surface where the optimum level of the variable for the response can easily be determined.

However Fig 3b exhibits a rather flat surface demonstrating that increase in substrate concentration and NaCl did not have much impact on enzyme production when time was a constant factor.

The enzyme production in the initial unoptimized conditions was found to be 1955 U/g, while after optimization, the maximum production was 4818 U/g after 84h when the substrate concentration was 25% with NaCl concentration at 0.65%.

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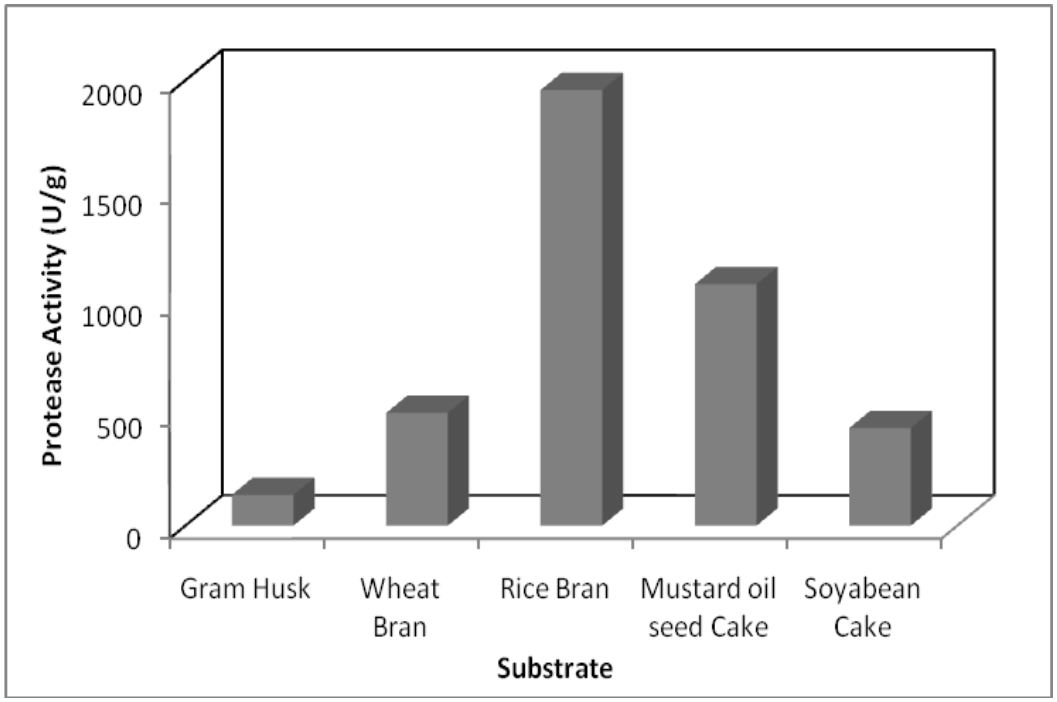

Figure 1: Protease production with different substrates (Gram husk, Wheat bran, Rice bran Mustard oilseed cake and Soybean cake)

Table 1a: Design Plackett Burman

Factor	1	2	3	4	5	6	7	8	9	10	11	Response
Std	A: Subs conc	B: Part. Size	C: Moist. Cont	D: Ino. Size	E: Time	F: KH_2PO_4	G: K_2HPO_4	H: KNO_3	J: NaCl	K: $MgSO_4$	L: $CaCl_2$	Protease Activity
	%	mm	ratio	%	h	%	%	%	%	%	%	U/g
1	10	2	2	3	72	1	0.1	0.1	0.1	0.5	0.01	1275
2	5	2	5	1	72	1	1	0.1	0.1	0.1	0.05	980.06
3	10	0.5	5	3	24	1	1	0.5	0.1	0.1	0.01	981
4	5	2	2	3	72	0.1	1	0.5	1	0.1	0.01	1075.5
5	5	0.5	5	1	72	1	0.1	0.5	1	0.5	0.01	1276.87
6	5	0.5	2	3	24	1	1	0.1	1	0.5	0.05	1143.25
7	10	0.5	2	1	72	0.1	1	0.5	0.1	0.5	0.05	1082
8	10	2	2	1	24	1	0.1	0.5	1	0.1	0.05	956
9	10	2	5	1	24	0.1	1	0.1	1	0.5	0.01	1162.67
10	5	2	5	3	24	0.1	0.1	0.5	0.1	0.5	0.05	888.25
11	10	0.5	5	3	72	0.1	0.1	0.1	1	0.1	0.05	1656.5
12	5	0.5	2	1	24	0.1	0.1	0.1	0.1	0.1	0.01	788.77

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Table 1b. Ranking of the variables investigated in the Plackett–Burman design.

Variable	Component	M_i^+–	M_i^-	$E(x_i)$
A	Substrate concentration	7113.17	6152.7	160.078
B	Particle Size	6337.48	6928.39	-98.485
C	Moisture Content	6945.35	6320.52	104.138
D	Inoculum Size	7019.5	6246.37	128.855
E	Time	7345.93	5919.94	237.665
F	KH_2PO_4	6612.18	6653.69	-6.9183
G	K_2HPO_4	6424.48	6841.39	-69.485
H	KNO_3	6259.62	7006.25	-124.44
J	NaCl	7370.79	5995.08	229.285
K	$MgSO_4$	6828.04	5795.58	172.077
L	$CaCl_2$	6706.06	6559.81	24.375

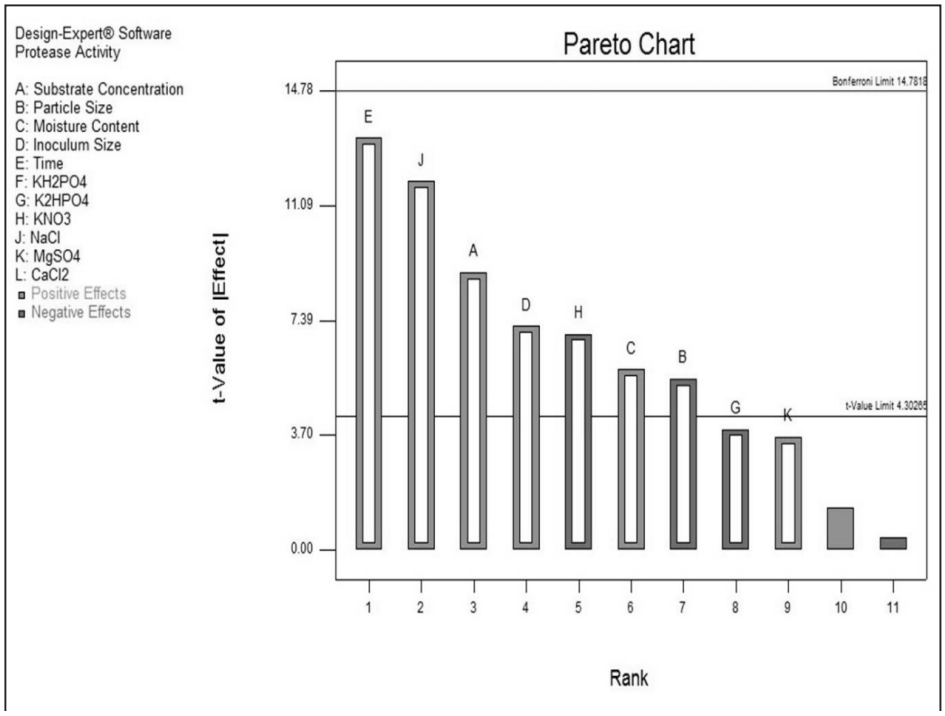

Figure 2: Pareto chart showing the relative effect of various factors on enzyme production

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Table 2: ANOVA Analysis of variance table for Plackett Burman

	Sum of		Mean	F	p-value	
Source	Squares	Df	Square	Value	Prob > F	
Model	567020	9	63002.2	65.42	0.0151	*significant*
A-Substrate Concentration	76875.2	1	76875.2	79.83	0.0123	
B-Particle Size	29097.9	1	29097.9	30.22	0.0315	
C-Moisture Content	32534.4	1	32534.4	33.78	0.0283	
D-Inoculum Size	49810.8	1	49810.8	51.72	0.0188	
E-Time	169454	1	169454	176	0.0056	
G-K_2HPO_4	14484.5	1	14484.5	15.04	0.0605	
H-KNO_3	46454.7	1	46454.7	48.24	0.0201	
J-NaCl	135620	1	135620	140.8	0.0070	
K-$MgSO_4$	12688.7	1	12688.7	13.18	0.0682	
Residual	1926.01	2	963.006			
Cor Total	568946	11				

Std. Dev.- 31.03; R-Squared - 0.9966; Adj R-Squared - 0.9814; Pred R-Squared - 0.8781; Adeq Precision – 29.771

Table 3 a: Design Summary CCD

Study Type			Response Surface			Runs			20	
Design Type			Central Composite			Blocks			No Blocks	
Design Model			Quadratic			Build Time (ms)			4.61	
Factor	Name	Units	Type	Subtype	Minimum	Maximum	-1 Actual	+1 Actual	Mean	Std. Dev.
A	Substrate Concentration	%	Numeric	Continuous	0.22689	50.22689	10	40	25	12.39514
B	Time	h	Numeric	Continuous	23.45546	144.5445	48	120	84	29.74835
C	NaCl	%	Numeric	Continuous	-0.35908	1.659076	0.05	1.25	0.65	8.2634298
Response Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Protease Activity	U/g	20	Polynomial	1413	4818	3325.95	1227.597	3.409766	None	Quadratic

Table 3 b: Central composite design matrix, response and predicted responses for protease activity

	Factor 1	Factor 2	Factor 3	Response 1		
Std Run	A:Substrate Concentration	B:Time	C:NaCl	Protease Activity (U/g)		
	%	H	%	Actual	Predicted	Residual
1	10	48	0.05	1413	1381.10	31.89
2	40	48	0.05	3883.5	3933.54	-50.04
3	10	120	0.05	1612.5	1549.58	62.91
4	40	120	0.05	3429	3375.27	53.72
5	10	48	1.25	2652	2736.39	-84.39
6	40	48	1.25	1981.5	2075.08	-93.58
7	10	120	1.25	3840	3820.62	19.37
8	40	120	1.25	2370	2432.56	-62.56

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Table 3 b: Central composite design matrix, response and predicted responses for protease activity (contd.)

	Factor 1	Factor 2	Factor 3	Response 1		
Std Run	A:Substrate Concentration	B:Time	C:NaCl	Protease Activity (U/g)		
9	0.23	84	0.65	1927.5	1960.00	-32.50
10	50.23	84	0.65	3015	2939.12	75.87
11	25	23.46	0.65	1920	1818.17	101.82
12	25	144.54	0.65	2202	2260.45	-58.45
13	25	84	-0.36	3612	3685.34	-73.34
14	25	84	1.66	4149	4032.28	116.71
15	25	84	0.65	4752	4753.24	-1.24
16	25	84	0.65	4816.5	4753.24	63.25
17	25	84	0.65	4741.5	4753.24	-11.74
18	25	84	0.65	4632	4753.24	-121.24
19	25	84	0.65	4818	4753.24	64.75
20	25	84	0.65	4752	4753.24	-1.24

Table 4: ANOVA (Analysis of variance) for Response Surface Quadratic Model

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	28539724	9	3171080	340.3412	< 0.0001	*Significant*
A-Substrate Concentration	1157236	1	1157236	124.2022	< 0.0001	
B-Time	236128.6	1	236128.6	25.34288	0.0005	
C-NaCl	145291	1	145291	15.59359	0.0027	
AB	264082.8	1	264082.8	28.3431	0.0003	
AC	5164095	1	5164095	554.2446	< 0.0001	
BC	419299	1	419299	45.00193	< 0.0001	
A^2	9559957	1	9559957	1026.037	< 0.0001	
B^2	13268111	1	13268111	1424.021	< 0.0001	
C^2	1441127	1	1441127	154.6712	< 0.0001	
Residual	93173.57	10	9317.357			
Lack of Fit	70147.07	5	14029.41	3.046363	0.1234	*not significant*
Pure Error	23026.5	5	4605.3			
Cor Total	28632897	19				

Std. Dev.- 96.53; R-Squared- 0.9967; Adj R-Squared - 0.9938;Pred R-Squared - 0.9801; Adeq Precision – 49.405

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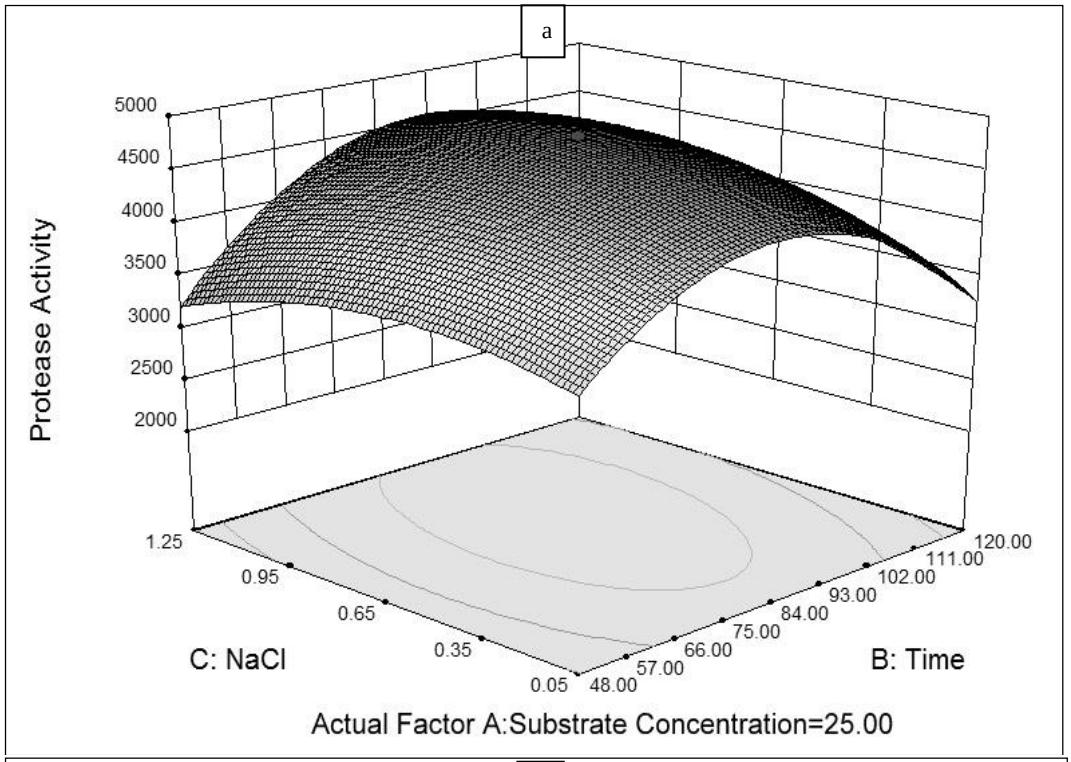

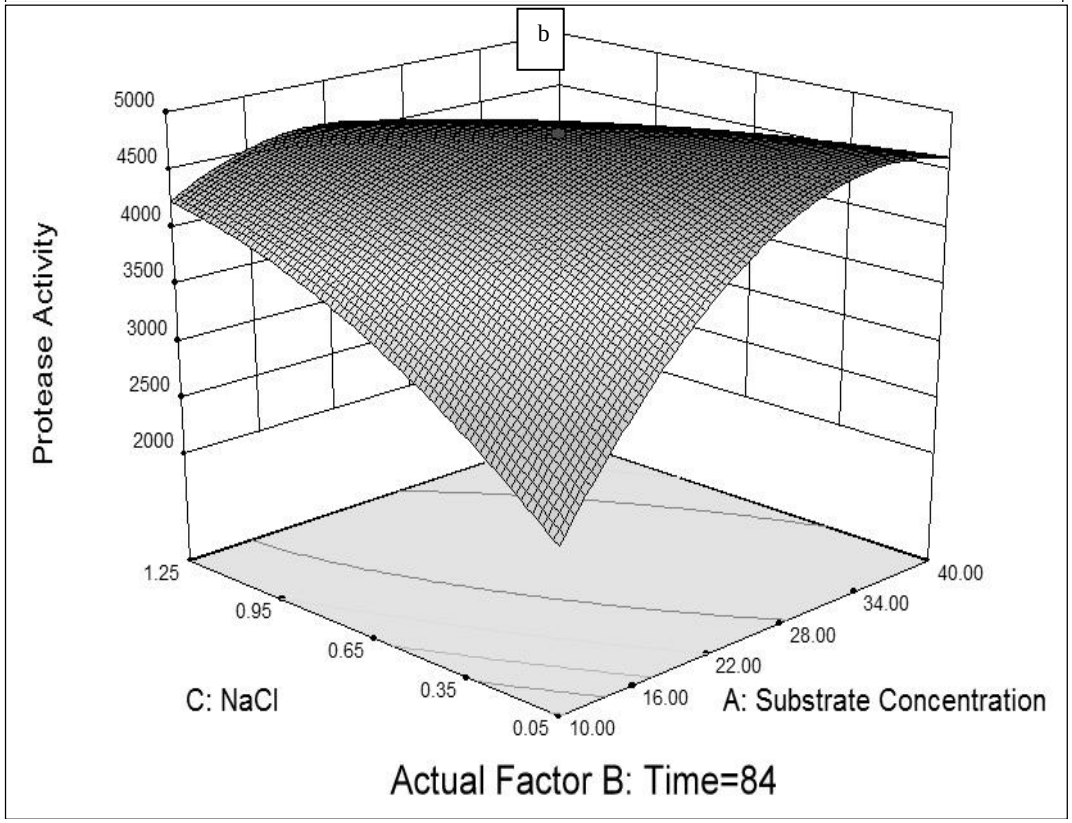

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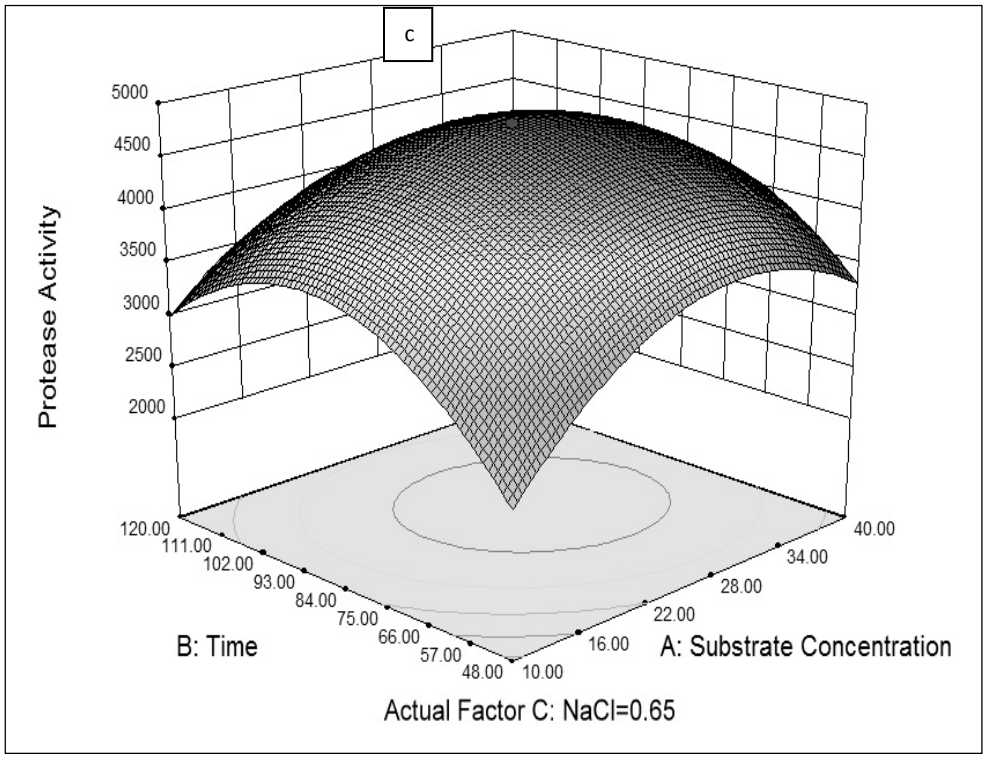

Figure 2. Contour plots of enzyme activity as a function of the interactions of two variables by keeping the other at centre level. (a) interactions of NaCl and time with substrate at 25% (b) interactions of NaCl and substrate with time at 84h (c) interactions of substrate and time with NaCl at 0.65%

DISCUSSION

A range of agro-industrial residues as cereal brans, gram husks, oilcakes, sugarcane baggase etc. have been used as low cost substrates for protease production in solid state fermentation.[20]. All the agricultural residues contain fat, crude fiber, carbohydrate, and protein, however, rice bran proteins are richer in albumin and lysine content and more easily digested than any other cereal bran proteins [21] This explains the better utilization of rice bran for protease production. Rice bran also contains minerals such as potassium, phosphorus and calcium, and hence it can be used as a substrate without further supplementation and it is a cheap agricultural residue easily available throughout the globe [10,22]. Considering the availability and properties, rice bran was chosen as substrate for further optimization.

Factors as substrate concentration, time, moisture etc. have been known to affect enzyme production [9,23]. In the present study substrate concentration, time, moisture and inoculum size had positive effect on the enzyme production. Higher concentrations of substrate in fermentation SSF provides a rich nutritional environment to the microorganism resulting in enhanced enzyme production [24]. The maximum enzyme production was observed in the stationary phase, the moisture level required was about 33% and a high inoculum size supported enzyme production. *Bacillus* strains usually produce secondary metabolites in late exponential and stationary phase [25]. The moisture levels in SSF processes vary between 30-85% and have a marked effect on growth kinetics of the microorganism which in turn affects enzyme production [26], while low inoculum size results in a lower number of cells in the production medium requiring a longer time to grow to an optimum number to utilize the substrate for the formation of the desired product [6]. In solid-state fermentation process, the availability of surface area play a vital role for microbial attachment, mass transfer of various nutrients and substrates and subsequent growth of microbial strain and product production [9]. In our study also smaller particle size supported the enzyme production. For smaller particles the surface area for growth is more, hence they are more suited for better enzyme production,

while surface area for growth is less and the interparticle space is more for larger particles [27].

Mineral salts as NaCl, $MgSO_4$, and $CaCl_2$ exhibited positive while KH_2PO_4, K_2HPO_4 and KNO_3 showed negative effect on the enzyme production. The effect of metal ions on protease production has been thoroughly investigated in many studies, where some enhance the production, while some inhibit [20]. This concludes that the effect of any metal or mineral on protease production depends on the unique physiological property of the microorganism and the type of the substrate being used.

The second step optimization demonstrated interactive effect of factors namely substrate concentration, time and NaCl. The contour plots are more helpful in understanding interaction and effect of two factors with the third factor fixed and evaluating the optimal level of each variable for maximum enzyme production. The curvature of the contours clearly established the optimal level of the variable, while the flat surface demonstrated the stability of the two factors with respect to one another factor in a wide range. This also establishes that the nutritional and microbial physiology play vital role in metabolite production when it comes to stability and optimum utilization of a production factor [23].

CONCLUSION

This work demonstrates the production optimization of a novel metalloprotease from a new *Bacillus* strain. Metalloprotease have applications in various industries along with medical applications in therapeutics and pathogenesis [16]. The work successfully employs statistical steps comprising of Plackett Burman and central composite factorial design to determine the optimum conditions that contribute the maximum enzyme production. Statistical optimization allows maximum amount of information while limiting the numbers of individual experiments required. A 2.5 fold increase in the enzyme production was observed during optimization process. An increase in 2-3 fold of product yield is always expected with SSF when compared to that of submerged fermentation [28]. Owing to the novel properties of the enzyme, the increase in production after optimization would help in the industrial exploitation of the enzyme. The study also demonstrates the utilization of the agricultural crop residues for the low cost production of the products as enzymes.

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